resultant PCR™ products were digested with the restriction enzymes *Bbu*I and *Age*I. The restricted DNA fragments were resolved by agarose gel electrophoresis in 1X TAE and the amplified *cry1C* fragment was purified using the Geneclean II® procedure. Similarly, plasmid pEG345 was digested with the restriction enzymes *Bbu*I and *Age*I, resolved by agarose gel electrophoresis in 1X TAE and the pEG345 vector fragment purified using the Geneclean II® procedure. The purified DNA fragments were ligated together using T4 ligase and used to transform *E. coli* DH5α using a standard transformation procedure. Transformants were selected on Luria plates containing 50 μg/ml Amp. Approximately 50% of the DH5α transformants generated by the R148 mutagenesis had lost the *Asu*II site, indicating that the mutagenic oligonucleotide primer E had been incorporated into the *cry1C* gene. Plasmid DNA from one transformant was used to transform *B. thuringiensis* EG10368 to Cml^R, using the electroporation procedure described by Mettus and Macaluso (1990). Transformants were selected on Luria plates containing 3 ug/ml chloramphenicol. One of the transformants was designated EG11822.

The arginine residue at amino acid position 148 was also replaced with random amino acids. This mutagenesis of R148 employed the mutagenic primer I (SEQ ID No: 53), the flanking primers H (SEQ ID NO:52) and F (SEQ ID NO:20), and plasmid pEG345 as the source of the *cry1C* DNA template. Primer I was also designed to eliminate an *Asu*II site within the wild-type *cry1C* sequence:

Primer I: (SEQ ID NO:53)

5'-GGGCTACTTGAAAGGGACATTCCTTCGTTTNNNATTTCTGGATTTGAAGTACCCC-3'

N (31,32,33) = 25% A, 25% C, 25% G, 25% T

cry1C template DNA was obtained from a PCR™ using the opposing primers H and F and plasmid pEG345 as a template. This DNA was then used as the template for a PCR™-mediated mutagenesis reaction that employed the flanking primers H and F and the mutagenic oligonucleotide I, using the procedure described by Michael (1994). The resultant PCR™ products were digested with the restriction enzymes *Bbu*I and *Age*I. The restricted DNA fragments were resolved by agarose gel electrophoresis in 1X TAE and the amplified *cry1C* fragment was purified using the Geneclean II® procedure. Similarly, plasmid pEG345 was digested with the restriction enzymes *Bbu*I and *Age*I, resolved by

agarose gel electrophoresis in 1X TAE and the pEG345 vector fragment purified using the Geneclean II® procedure. The purified DNA fragments were ligated together using T4 ligase and used to transform *E. coli* DH5α to ampicillin resistance using a standard transformation procedure. Transformants were selected on Luria plates containing 50 ug/ml ampicillin. The DH5α transformants were pooled together and plasmid DNA was prepared using the alkaline lysis procedure. Plasmid DNA from the DH5α transformants was used to transform *B. thuringiensis* EG10368 to Cml^R, using the electroporation procedure described by Mettus and Macaluso (1990). Transformants were selected that exhibited an opaque phenotype on starch agar plates containing 3 ug/ml chloramphenicol, indicating crystal protein production. Approximately 90% of the opaque EG10368 transformants generated by the R148 mutagenesis had lost the *Asu*II site, indicating that the mutagenic oligonucleotide primer I had been incorporated into the *cry1C* gene.

5.4 EXAMPLE 4 -- BIOASSAY EVALUATION OF CRY1C* TOXINS

EG10368 transformants containing mutant *cry1C* genes were grown in C2 medium, described by Donovan *et al.* (1988), for 3 days at 25°C or until fully sporulated and lysed. The spore-Cry1C crystal suspensions recovered from the spent C2 cultures were used for bioassay evaluation against neonate larvae of *Spodoptera exigua* and 3rd instar larvae of *Plutella xylostella*.

EG10368 transformants harboring Cry1C mutants generated by random mutagenesis were grown in 2 ml of C2 medium and evaluated in one-dose bioassay screens. Each culture was diluted with 10 ml of 0.005% Triton X-100® and 25 µl of these dilutions were seeded into an additional 4 ml of 0.005 % Triton X-100® to achieve the appropriate dilution for the bioassay screens. Fifty µl of this dilution were topically applied to 32 wells containing 1.0 ml artificial diet per well (surface area of 175 mm^2). A single neonate larvae (*S. exigua*) or 3rd instar larvae (*P. xylostella*) was placed in each of the treated wells and the tray was covered by a clear perforated mylar strand. Larval mortality was scored after 7 days of feeding at 28-30°C and percent mortality expressed as ratio of the number of dead larvae to the total number of larvae treated.

Three EG10368 transformants, designated EG11740, EG11746, and EG11747, were identified as showing increased insecticidal activity against *Spodoptera exigua* in replicated bioassay screens. The putative Cry1C variants in strains EG11740, EG11746, and EG11747 were designated Cry1C.563, Cry1C.579, and Cry1C.499, respectively. These three variants contain amino acid substitutions within the loop region between α helices 3 and 4 of Cry1C. EG11740, EG11746, and EG11747, as well as EG11726 (which contains the wild-type *cry1C* gene from strain EG6346) were grown in C2 medium for 3 days at 25°C. The cultures were centrifuged and the spore/crystal pellets were washed three times in 2X volumes of distilled-deionized water. The final pellet was suspended in an original volume of 0.005% TritonX-100 and crystal protein quantified by SDS-PAGE as described by Brussock and Currier (1990). The procedure was modified to eliminate the neutralization step with 3M HEPES. Eight δ-endotoxin concentrations of the spore/ crystal preparations were prepared by serial dilution in 0.005% Triton X-100 and each concentration was topically applied to wells containing 1.0 ml of artificial diet. Larval mortality was scored after 7 days of feeding at 23-30°C (32 larvae for each δ-endotoxin concentration). Mortality data was expressed as LC_{50} and LC_{95} values, in accordance with the technique of Daum (1970), the concentration of Cry1C protein (ng/well) causing 50% and 95% mortality, respectively (Table 5, Table 6, and Table 7). Strains EG11740 (Cry1C.563) and EG11746 (Cry1C.579) exhibited 3-fold lower LC_{95} values than the control strain EG11726 (Cry1C) against *S. exigua*, while retaining a comparable level of activity against *P. xylostella*. EG11740 and EG11746 also exhibited significantly lower LC_{50} values against *S. exigua*.